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Unveiling a Multiprotein Complex Involved in Excitation-Transcription Coupling in Skeletal Muscle

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Electrical activity regulates the expression of skeletal muscle genes by a process known as ''excitation-transcription'' (E-T) coupling. We have demonstrated that ATP and its metabolites released during depolarization activate membrane P2X/P2Y receptors and behave as fundamental mediators between electrical stimulation, slow intracellular calcium transients and gene expression. We propose that this signaling pathway would require the proper coordination between the voltage sensor (dihydropyridine receptor, DHPR), pannexin hemichanel (ATP release conduit), nucleotide receptors, and several signal transduction molecules. The goal of this study was to assess protein interactions between the E-T machinery in skeletal muscle, in order to unveil a putative signaling complex.

Co-immunoprecipitation of the selected proteins was achieved in extracts of newborn rat derived myotubes, and in rat and mouse adult muscle triad-enriched fractions. Protein components of multiprotein complexes were isolated using blue native SDS/PAGE. Immunofluorescence assays were performed in isolated fibers derived from mice adult muscles.

DHPR, $P2Y_2$ receptor, Panexin 1, phospholipase Cγ1 and dystrophin, all co-immunoprecipitated in a crossed manner in the different preparations assessed. DHPR, pannexin-1, $P2Y_2$ and $P2X_7$ did show a striated pattern of distribution by immunofluorescence, possibly representing location at the T-tubules in adult skeletal fibers. Using blue-native SDS/PAGE we detected that, in adult muscle triads, DHPR is an integral part of large multiprotein complexes of various sizes and we are currently looking for novel actors within these complexes using MALDI-TOF mass spectrometry.

Several proteins involved in the E-T coupling process appear to interact in skeletal muscle T-tubules, suggesting that a signaling complex other than that involved in E-C coupling is present.

FONDAP 15010006; AFM 14562, Conicyt 79090021, Fondecyt 11100454.

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P21-Activated Kinase-1 and Fty720 Alter Atrial Excitation-Contraction Coupling

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We previously reported that Pak1 participates in the integrated regulation of excitation-contraction coupling (e-cc) in adult rat ventricular myocytes via activation of protein phosphatase 2A (PP2A). Pak1 inhibited the ability of isoproterenol (ISO) to increase the peak of the Ca2+ transient in a single myocyte without altering the increase in shortening. Here we test the hypothesis that Pak1 signaling regulates atrial e-cc and potentiates arrhythmicity. Under normal conditions single adult rat atrial myocytes expressing constitutively active Pak1 (CA-Pak1) and paced at 0.5 Hz have a slower time constant of [Ca2+]i-transient decay compared to uninfected controls as assessed by confocal microscopy and fluo-4 fluorescence. Increase of the pacing frequency to 2.0 Hz resulted in a reduced peak of the [Ca2+]i-transient, an increase in diastolic [Ca2+]I, and more rapid [Ca2+]i decay compared to 0.5 Hz for both CA-Pak1 expressing myocytes and controls. Return to 0.5 Hz pacing restored these values to near controls, with some extra-systolic Ca2+ release. Stimulation with ISO (100 nM) of CA-Pak1-expressing myocytes paced at 3.0 Hz caused 20% missed beats that were subsequently restored on washout. Uninfected myocytes paced at 2.0 Hz in the presence of ISO had 1:1 fidelity of beating to stimulation, however treatment with fingolimod (FTY720, 25 nM) in addition to ISO caused 50% missed beats that were restored on washout of the drug. Cultured monolayers of rat neonatal myocytes expressing CA-Pak1 had a more rapid rate of spontaneous beating than controls that was resistant to further increase with ISO, as assessed by microelectrode array (MEA). We conclude that Pak1 and FTY720 act through the same pathways in atrial myocytes and induce arrhythmic activity under conditions of stress.

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Identification of a Chemical Supressor of Cardiac Arrhythmia Induced by Aberrant Calcium Homeostasis

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Ca^{2+} homeostasis is essential for rhythmic contractions of the adult myocardium. In the developing embryo, a role of Ca^{2+} in establishing cardiac rhythmicity has been proposed, but the underlying mechanism has yet to be fully explored. We have previously shown that zebrafish *tremblor* mutant embryos develop a fibrillating heart and that the *tremblor* locus encodes a cardiac-specific isoform of the Na^{+}/Ca^{2+} exchanger, NCX1h. While rhythmic Ca^{2+} waves were detected with each cardiac contraction in wild type hearts, only local, unsynchronized Ca^{2+} signals together with sporadic contractions were observed in *tremblor* hearts. These data suggest that loss of function of NCX1h induces cardiac fibrillation in *tremblor* and that *tremblor* mutants can serve as a model for cardiac arrhythmia induced by aberrant calcium homeostasis.

From a collection of small molecules, we found a novel compound, OK-F7, that could restore rhythmic cardiac contractions in *tremblor* embryos at low micromolar concentrations in a reversible and dosage-dependent manner. We tested the effect of OK-F7 on isolated cardiomyocytes under Ca^{2+} overload conditions and found that treatment with 1 μM OK-F7 reduces the frequency of spontaneously propagating Ca^{2+} waves by approximately half, while 25 μM OK-F7 almost entirely abolishes Ca^{2+} waves. A biochemical pull-down assay using an OK-F7 affinity column isolated a mitochondrial membrane protein that selectively bound to OK-F7. These findings suggest that OK-F7 suppresses cardiac fibrillation by reducing the frequency of arrhythmogenic Ca^{2+} waves in Ca^{2+}-overloaded cardiomyocytes and indicate a novel role of mitochondria in cardiac Ca^{2+} handling and control of rhythmicity. Further validation of the target protein and biophysical experiments confirming a role of mitochondria in the mechanism of OK-F7 action are underway and will be discussed.

Supported by NIH-1R01HL096980-01A1 and FWF-J3065-B11.

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Beta-Adrenergic Activation Enhances Histone Deacetylase 4 nuclear Localization via Pka and Counters Camkii-Dependent Effects in Adult Rabbit Cardiac Myocytes

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Nuclear localization of histone deacetylase 4 (HDAC4), that represses transcription and possibly heart failure by suppressing the transcription factor MEF2, is dependent upon its phosphorylation status. Phosphorylation at three key serines can trigger the nuclear export of HDAC4, while dephosphorylation results in nuclear accumulation. HDAC4 is known to bind CaMKII and phosphorylation by this kinase facilitates nuclear export. A probable PKA phosphorylation site has been identified, suggesting the hypothesis that HDAC4 localization is modulated by both CaMKII and PKA. We tested whether B-adrenergic-induced PKA activity alters HDAC4 localization in adult rabbit ventricular myocytes using confocal imaging and GFP-tagged HDAC4. At baseline, the nuclear to cytosolic ratio of HDAC4 (Nuc/Cyto) was $2.19 \pm .04$ (n=638). Myocytes treated with 1μM isoproterenol (Iso) had a $58 \pm 8\%$ increase in HDAC4 Nuc/Cyto compared to baseline (n=123). Activation of adenylyl cyclase via forskolin (10μM) produced a similar $50 \pm 5\%$ increase in Nuc/Cyto ratio (n=107). CaMKII inhibition at rest with 1μM KN-93 also raised the Nuc/Cyto by $40 \pm 6\%$ (n=99), whereas PKA inhibition (2 μM H-89) did not alter baseline HDAC4 localization (n=91). Conversely, phosphatase inhibition (1 μM okadaic acid) decreased basal Nuc/Cyto by $57 \pm 5\%$ (n=97). Thus, baseline CaMKII activity limits the degree of nuclear HDAC4 localization, and preventing HDAC4 dephosphorylation exacerbates this. Furthermore, B-adrenergic activation causes HDAC4 nuclear import via PKA (vs other cAMP or B-adrenergic signaling). In conclusion, while CaMKII activates HDAC4 nuclear export, B-adrenergic signaling via PKA favors nuclear retention/import and transcriptional repression in cardiac hypertrophic signaling.

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Could Epac Modulate Ca^{2+} Handling At Different Intramyocyte Microdomains?

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The universal second messenger 3',5'-cyclic adenosine monophosphate (cAMP) has been considered for many years the main regulator of several cellular physiopathological process uniquely via protein kinase A (PKA) activation. However, in the last years the discovery of a new sensor for cAMP named Epac (exchange protein directly activated by cAMP) has profoundly changed this cAMP-PKA dogma. In this sense, Epac is able to modulate Ca2+ handling and induce hypertrophy independently of PKA in cardiomyocytes. Epac has been immunolocalized in the cytosol and the perinuclear area in cardiac myocytes infected with Epac1 adenovirus. In fact, several evidences point that physiological functions of Epac may be profoundly linked to its specific intracellular localization and the proximity with its molecular partners. We analyzed endogenous Epac localization by immunolabeling in adult cardiomyocytes and found a preferential perinuclear localization. Then we analyzed Epac actions on Ca2+ handling at different subcellular microdomains (cytosol and nucleus) in isolated rat ventricular myocytes. To simultaneously and separately analyze Epac effects on intracellular and intranuclear Ca2+ concentration ([Ca2+]i and [Ca2+]n respectively), fluo-3 loaded adult ventricular myocytes field-stimulated at 1 Hz were subjected to line scan imaging through the nucleus. Epac was activated by 10 μM 8-p-CPT (specific Epac activator) in the absence or presence of various antagonists. We observed that unlike the